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Review Letter

Phospholipid flippases

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Protein mediated phospholipid translocation through membranes has been observed in rat liver endoplasmic reticulum and in the plasma membrane of erythrocytes as well as in a few other cell membranes. Lipid translocation in plasma membranes is ATP dependent and selectively accumulates aminophospholipids on the inner monolayers.

1. INTRODUCTION

Phospholipids in biological membranes form bilayers. Such structures normally allow only very slow exchange of lipids between the two leaflets, with a $\tau_{1/2}$ of flip-flop of the order of a day or more. This stability is certainly beneficial for the cohesion of biological membrane structures; however, it creates certain problems of lipid distribution. Firstly, lipids are synthesized asymmetrically on the endoplasmic reticulum of eukaryotes or on the plasma membranes of prokaryotes and must be redistributed rapidly otherwise these membranes will collapse due to exaggerated imbalance between the two leaflets. Secondly, the compositional asymmetry of plasma membranes has been demonstrated but does not correspond to the asymmetrical production of lipids in these membranes. Thus, lipid asymmetry in plasma membranes must be formed and afterwards maintained by some process which overcomes long term randomization. These tasks could be assigned to specific 'phospholipid flippases', as first proposed by Bretscher in 1973 [1].

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2. 'PHOSPHOLIPID FLIPPASES' IN EUKARYOTIC INNER MEMBRANES AND IN PROKARYOTIC CELLS

In eukaryotic cells, the enzymes of glycerolipid biosynthesis are principally located on the endoplasmic reticulum and exclusively on its cytoplasmic surface. To maintain a phospholipid bilayer in this organelle, newly synthesized phospholipids must be translocated to the lumenal surface. The same problem arises in bacteria where lipid must be rapidly exported from the inner surface to the outer surface of the bacterial membrane. Consistent with this, rapid transmembrane movement of lipids has been found in microsomal membranes and in the plasma membrane of *Bacillus megaterium* (see the review by Op den Kamp [2] and references cited therein). These data suggest involvement in such membranes of a phospholipid translocating protein.

In 1985, Bishop and Bell [3] showed that a short-chain water soluble phosphatidylcholine (dibutyroyl-PC) and the corresponding lyso-derivative are rapidly transported through rat liver endoplasmic reticulum. The transport is saturatable and inhibited by proteases, protein modification reagents and structural analogs. This suggests that microsomal transport of phosphatidylcholine (PC) and lyso PC is indeed mediated by a protein. Recently

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the PC transporter has been successfully reconstituted into proteoliposomes by Backer and Dawidowicz [4], using detergent-solubilized protein from rat liver microsomes. However, the latter authors found no effect of trypsin or *N*-ethylmaleimide. The specificity of the PC-flippase in microsomes has in fact not been investigated thoroughly; this protein does not seem to require ATP and may work in both directions, stimulating equally inner and outer lipid flux. Perhaps the mechanism of lipid translocation is the formation of local non bilayer structures [5]. If such is the case, the protein works like a valve allowing lipid equilibration between inner and outer surfaces. Such proteins should be looked for in bacteria membranes where genetic manipulations are available.

3. PHOSPHOLIPID ASYMMETRY IN EUKARYOTIC PLASMA MEMBRANES

Phospholipid transverse asymmetry has been well documented in the erythrocyte membrane and several other plasma membranes [2]. This compositional asymmetry was demonstrated by chemical derivatization and enzymatic hydrolysis using exogenous phospholipases. The choline derivatives, PC and sphingomyelin (SM), are found principally in the outer monolayer, while the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS) are found principally in the inner monolayer. Although the characterization of the lipid asymmetry is more difficult in the plasma membrane of cells containing organelles, the general conclusion, inferred from studies on platelets and fibroblasts, is again that the aminolipids are located in the inner layer [2].

This asymmetry could be the result of vectorial membrane biogenesis. However, for cells such as erythrocytes with a low metabolic activity and slow lipid turnover, this stable asymmetry which lasts during the 120 days of the cell's life span, appears to be incompatible with transverse diffusion rates of phospholipids as determined in model systems. The interaction of the cytoskeleton proteins with the aminophospholipids was suggested by Haest and collaborators [6] as an explanation of the maintenance of lipid asymmetry in erythrocytes. But there is now compelling evidence that in addition, or perhaps as a sole cause of lipid asymmetry, a specific ATP driven phospholipid flippase, also called aminophospholipid translocase, continuously pumps the aminolipids from the outer to the inner monolayer.

4. THE AMINOPHOSPHOLIPID TRANSLOCASE IN HUMAN ERYTHROCYTES

Protein mediated lipid translocation in erythrocytes was discovered in 1984, using spin-labeled phospholipids with a short β-chain bearing a nitroxide radical [7]. The aminophospholipids (PS and PE) were found to flip rapidly ($\tau_{1/2}$ respectively of 5 min and 1 h at 37°C) while the choline derivatives (PC and SM) require much longer times ($\tau_{1/2} > 10$ h at 37°C). A requisite for the rapid diffusion of aminophospholipids is the presence of hydrolysable intracellular ATP. Furthermore the transport is inhibited by protein reagents such as *N*-ethylmaleimide and iodoacetamide or ATPase inhibitors such as vanadate ions but not ouabain. It is also inhibited by micromolar concentrations of cytosolic calcium. These observations led us to postulate the existence of a specific carrier protein for the outside-inside transport of aminophospholipids in human erythrocytes [7].

Using the spin label assay, apparent K_m values of respectively 5 μM and 50 μM were determined for the PS and PE analogues. It was also shown that PS and PE compete for the same transport site but that PS has a higher affinity, in agreement with its lower apparent K_m. Experiments with ghosts resealed in the presence of variable amounts of ATP have shown that the initial rate of aminophospholipid translocation is a function of ATP concentration. The K_m for ATP is of the order of 1.3 mM [8,9].

This ATP-dependent transmembrane segregation between phospholipids in red cells was confirmed by several totally independent techniques. For example Daleke and Huestis [10], using cell morphology as an assay for exogenous phospholipid distribution, concluded that the incorporation of aminophospholipids was protein mediated and involved an ATPase. In 1986, Tilley et al. [11] incorporated long chain radioactive phospholipids by a non-specific phospholipid carrier protein and tested their transmembrane

distribution by the phospholipase A_2 technique. These authors showed the selective transport of aminophospholipids from the outer to the inner monolayer of human erythrocytes; the requirement for cytosolic ATP was then confirmed. Finally, Connor and Schroit [12] demonstrated by fluorescence energy transfer the selective translocation of fluorescent aminophospholipids in erythrocytes, while Morrot et al. [13] took advantage of the difference of viscosity between the two leaflets of the erythrocyte membrane and showed by photobleaching measurements that fluorescent long chain PS molecules are preferentially located on the inner monolayer.

Because the equilibrium distribution does not correspond to all the aminolipids being on the inner leaflet, this equilibrium must be a steady state with continuous inward and outward diffusion of aminophospholipids. The outward motion could be a passive leakage through the lipid bilayer. However, recent results from our laboratory have shown that the half time for inside-outside PS translocation is smaller than one would expect for a simple lipid diffusion; it is also smaller for PS and PE than for PC and SM, and finally, this accelerated outward motion is inhibited by protein reagents such as *N*-ethylmaleimide or by cytosolic Mg^{2+} depletion [14]. Therefore, the outward motion is apparently also protein mediated. Possibly the same 'flippase' accelerates the mobility of aminolipids in both directions but with a higher efficiency under normal conditions for outside inside transport, thereby creating the aminophospholipid asymmetry. Note that in order to explain the accumulation of PC and SM in the outer monolayer, it is not necessary to postulate the existence of another enzyme specific for choline derivatives: a mere counter diffusion process can explain the observation that the non-aminolipids occupy mostly the outer monolayer. In addition the stability of the sphingomyelin molecules on the outer monolayer is probably enhanced by hydrogen bonds between these molecules [15].

The molecular mechanism underlying specific aminolipid translocation is unknown. We can eliminate such mechanisms as electric and curvature effects. The former effect should be ruled out because the discrimination between the lipids is not based on their charge, for example PA flips approximately like PC; besides the potential is negative inside which is unfavourable for PS translocation. As for membrane curvature, which is responsible for lipid asymmetry in sonicated vesicles, it is unlikely to be important in erythrocytes because the translocase activity is identical in echinocytes, discocytes or stomatocytes [10]. A reasonable model for the aminophospholipid translocase would probably resemble that of an ATP-dependent ion pump. The protein (or proteins) involved would have a single site with a high affinity for aminophospholipid on the extracellular surface; phosphorylation would induce a conformation change exposing the selected lipid to the internal face where the binding affinity would be low. Recently, radioiodinated photoactivatable PS as well as radioactive SH reagents have been used by two laboratories tentatively to label the protein(s) corresponding to the aminophospholipid translocation of human erythrocytes [16,17]. According to Connor and Schroit [17], the flippase appears to be a 31 kDa protein, which is labeled by the two families of covalent probes. However, several other bands are also labeled by these probes and direct proof of the involvement of this particular protein is still awaited.

5. AMINOPHOSPHOLIPID TRANSLOCASE IN THE PLASMA MEMBRANE OF OTHER CELLS

From the results of experiments with spin labels, it appears that the same type of protein is present not only in red cells of humans but also of cows, sheep, rats and hence, probably, most mammals. The selective translocation of aminophospholipids through other plasma membranes has been demonstrated with spin-labels, for example in platelets and lymphocytes [18,19]. Platelet shape change analysis led to the same conclusion [10]. Using the observation of the redistribution of fluorescent phospholipids within the internal membranes of a single cell, Martin and Pagano [20] have concluded that a similar mechanism of ATP-dependent aminophospholipid translocation may exist in the plasma membrane of cultured fibroblasts. These observations were made at low temperature, which resulted in the selective internalization of the aminophospholipids; they cannot be explained by endocytosis.

In conclusion there is now strong experimental support for the concept of a rather universal ATP-dependent membrane bound enzyme which can control the transmembrane distribution of phospholipids in the plasma membrane of eukaryotic cells. Whether such an enzyme also exists in prokaryotic cells has yet to be determined.

6. POSSIBLE BIOLOGICAL FUNCTIONS OF THE AMINOPHOSPHOLIPID TRANSLOCASE

The physiological utility of an asymmetric lipid distribution in plasma membranes is not completely elucidated. Several non exclusive hypotheses have been put forward.

6.1. *Influence on cell shape*

Cell shapes are due to the cumulative effects of several constraints; one is the curvature of the bilayer imposed by the relative areas of the two bilayer leaflets, another is the adjustment of the membrane to the cytoskeletal network. Because the aminophospholipid translocase is responsible for the actual lipid distribution at equilibrium, it certainly plays a role in erythrocyte and platelet cell shape [7,10] and probably for other cells too.

6.2. *Cell ageing*

Aged cells lose their lipid asymmetry either because of the effects on the translocase of increased cytosolic Ca^{2+} or lipid peroxidation or both. PS located on the outer layer of erythrocytes provokes an enhanced binding to macrophages as shown in in vitro experiments [21]. Thus the control of lipid asymmetry is one of the requirement for cell survival in the blood stream [22].

6.3. *Control of kinase C activity*

PS differentially modulates protein kinase C and auto-phosphorylation [22]. Thus fluctuations in the PS content of the plasma membrane due for example to Ca^{2+} influx, may affect protein kinase C activity.

6.4. *Triggering of endocytosis*

Endocytosis requires the bending of cell surface with, locally, an accumulation of lipids on the inner leaflet. Consequently the aminophospholipid translocase could be involved in ATP-dependent endocytosis.

6.5. *Platelet stimulation*

It has been reported that platelet stimulation is accompanied by PS relocation in the outer monolayer of the plasma membrane [24]. To what extent this phenomenon is essential in the triggering of the cascade of events involved in blood coagulation has yet to be determined.

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